Steps involved in activation of the complex of pro-matrix metalloproteinase 2 (progelatinase A) and tissue inhibitor of metalloproteinases (TIMP)-2 by 4-aminophenylmercuric acetate

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Tissue inhibitor of metalloproteinases (TIMP)-2 forms a non-covalent complex with the precursor of matrix metalloproteinase 2 (proMMP-2, progelatinase A) through interaction of the C-terminal domain of each molecule. We have isolated the proMMP-2–TIMP-2 complex from the medium of human uterine cervical fibroblasts and investigated the processes involved in its activation by 4-aminophenylmercuric acetate (APMA). The treatment of the complex with APMA-activated proMMP-2 by disrupting the Cys^{73}–Zn^{2+} interaction of the zymogen. This is triggered by perturbation of the proMMP-2 molecule, but not by the reaction of the SH group of Cys^{73} with APMA. The 'activated' proMMP-2 (proMMP-2*) formed a new complex with TIMP-2 by binding to the N-terminal inhibitory domain of the inhibitor without processing the propeptide. Thus the APMA-treated proMMP-2*–TIMP-2 complex exhibited no gelatinolytic activity. In the presence of a small amount of free MMP-2, however, proMMP-2* in the complex was converted into the 65 kDa MMP-2 by proteolytic attack of MMP-2, but the complex did not exhibit gelatinolytic activity. The gelatinolytic activity detected after APMA treatment was solely derived from the activation of free proMMP-2. The removal of the propeptide of the proMMP-2* bound to TIMP-2 was also observed by MMP-3 (stromelysin 1), but not by MMP-1 (interstitial collagenase). MMP-3 cleaved the Asn^{80}–Tyr^{81} bond of proMMP-2*. On the other hand, when MMP-3 was incubated with the proMMP-2–TIMP-2 complex, it bound to TIMP-2 and rendered proMMP-2 readily activatable by APMA. These results indicate that the blockage of TIMP-2 of the complex with an active MMP is essential for the activation of proMMP-2 when it is complexed with TIMP-2.

INTRODUCTION

Matrix metalloproteinase 2 (MMP-2, gelatinase A) is a member of the matrixin family [1]. The enzyme readily digests denatured collagens, but also degrades a number of extracellular matrix components, including collagens IV, V, VII and XI, fibronectin, laminin, elastin (see [1,2] for reviews), aggrecan core protein [3] and cartilage link protein [4]. Many cell types in culture constitutively synthesize and secrete the enzyme [5,6], but an elevated expression of MMP-2 in a number of cancer cells and their surrounding stromal cells (see [7] for a review) and in corneal stroma after keratectomy [8] suggests that the enzyme may participate in tumour-cell invasion and metastasis as well as in tissue remodelling.

Like other MMPs, MMP-2 is synthesized and secreted from the cells as an inactive precursor (proMMP-2) of 72 kDa. Thus activation of proMMP-2 is a crucial step for the expression of its enzymic activity. ProMMP-2 comprises a propeptide domain, a catalytic domain containing three repeats of fibronectin type II-like motifs, and a C-terminal hemopexin-like domain [5]. Latency of proMMP-2 is thought to be retained through the co-ordination of the Zn atom at the active site and the SH group of the cysteine residue in the highly conserved PRCGNPD sequence of the propeptide [9,10]. ProMMP-2 is readily activated *in vitro* by 4-aminophenylmercuric acetate (APMA) [5,6,11]. This is thought to result from disruption of the Cys^{73}–Zn interaction by the reaction of APMA with the SH group of Cys^{73} [9,10]. ProMMP-2 is resistant to activation by many proteinases [6], although Crabbe et al. [12] have recently reported that MMP-7 (matrilysin) slowly activates proMMP-2 up to 64% of the APMA-activated MMP-2 activity. Another potential physiological activator of proMMP-2 has been found on the plasma membrane of normal or neoplastic cells treated with concanavalin A [13,14] or phorbol myristate acetate [15,16]. Recently, Sato et al. [17] isolated a cDNA clone of a new membrane-type MMP (MT-MMP) and characterized the recombinant enzyme as proMMP-2 activator. However, the exact mechanism of proMMP-2 activation by this membrane component is not known. An additional complexity in the process of proMMP-2 activation has been introduced as proMMP-2 was shown to bind to an endogenous tissue inhibitor of metalloproteinases (TIMP)-2 [18,19] through the C-terminal domains of these two molecules [20–23]. In this complex, the N-terminal inhibitory domain of TIMP-2 is unmasked, so that the complex is able to inhibit active MMPs [24]. A number of investigators have studied the activation of proMMP-2–TIMP-2 complex by APMA and have reported that the formation of proMMP-2–TIMP-2 complex does not prevent its activation by APMA; the activated complex exhibits gelatinolytic activity equivalent to about 10% of MMP-2 [18–20,23–27]. It was also reported that this activation was accompanied by a shift in molecular mass of the 72 kDa proMMP-2 to 65–62 kDa [19,20,24–26] and the enzymic activity was inhibited by an additional stoichiometric amount of TIMP-2 [19,26]. Thus the fate of the TIMP-2 in the complex upon APMA treatment is not clearly understood.

In the present study we have purified the proMMP-2–TIMP-2 complex from the culture medium of human uterine cervical fibroblasts and investigated the possible molecular rearrange-

Abbreviations used: α_2M, α_2-macroglobulin; APMA, 4-aminophenylmercuric acetate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; MMP, matrix metalloproteinase; proMMP-2*, APMA-activated proMMP-2 of 72 kDa; MT-MMP, membrane-type MMP; TIMP, tissue inhibitor of metalloproteinases; PVDF, poly(vinylidene difluoride); PTH, phenylthiohydantoin.

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ments of proMMP-2 and TIMP-2 in the complex that may take place upon APMA activation. In contrast with previous findings by others [18–20,23–27], our results demonstrate that the treatment of the complex with APMA does not produce any gelatinolytic activity. This is due to rapid binding of the N-terminal domain of TIMP-2 to the activated proMMP-2 before the propeptide of proMMP-2 is proteolytically processed. Our studies also demonstrate that the initial step involved in proMMP-2 activation by APMA is not the reaction with the SH group of Cys^{73}, but results from the molecular perturbation of proMMP-2 induced by APMA.

EXPERIMENTAL

Materials

APMA, gelatin (porcine skin) and bovine trypsin were from Sigma. Dulbecco's modified Eagle's medium (DMEM), antibiotics, fetal-calf serum (FCS) and lactalbumin hydrolysate were from GIBCO. Human α_2-macroglobulin (α_2M) was kindly provided by Dr. J. J. Enghild at Duke University Medical Center (Durham, NC, U.S.A.). Human uterine cervical fibroblasts were kindly provided by Professor Y. Mori at the Tokyo College of Pharmacy (Tokyo, Japan). ProMMP-3 (prostromelysin-1) was purified from the culture medium of human synovial fibroblasts as described by Ito and Nagase [28]. Purified proMMP-3 was activated with chymotrypsin, and the 45 kDa MMP-3 was isolated by the method of Ogata et al. [29]. TIMP-1 was purified from the culture medium of HT-1080 cells as described by Morodomi et al. [30].

Cell cultures

Human cervical fibroblasts were cultured in DMEM containing 10% FCS. After confluency, the medium was changed to DMEM containing 5% FCS, and cells were cultured for 5–7 days. This conditioned medium was then harvested and used for purification of proMMP-2 and the proMMP-2–TIMP-2 complex.

Purification of proMMP-2 and the proMMP-2–TIMP-2 complex

ProMMP-2–TIMP-2 complex and proMMP-2 free of TIMP-2 were purified from culture medium of human uterine cervical fibroblasts. First, the culture medium was applied to a gelatin–Sepharose 4B column equilibrated with TNC buffer [50 mM Tris/HCl (pH 7.5)/0.15 M NaCl/10 mM $CaCl_2$/0.02% NaN_3]. The column was then washed with the same buffer containing 1% dimethyl sulphoxide, followed by elution of the enzyme with the same buffer containing 5% dimethyl sulphoxide. The latter peak was pooled, dialysed against TNC buffer, concentrated with an Amicon YM-10 membrane and subjected to gel-permeation chromatography on Sephacryl S-200. Earlier-eluted fractions contained the proMMP-2–TIMP-2 complex, while later fractions contained proMMP-2 free of TIMP-2.

Enzyme assays

All enzymic assays were carried out in TNC buffer containing 0.05% Brij 35. The gelatinolytic activity of MMP-2 was measured using 150 μg of heat-denatured [^{14}C]acetylated type I collagen (guinea pig) in the total volume of 200 μl as described by Harris and Krane [31]. One unit of gelatinolytic activity degraded 1 μg of gelatin/min at 37 °C. The activity of MMP-3 was measured against reduced, carboxy[^{3}H]methylated transferrin as described previously [32].

Determination of the amount of the proMMP-2–TIMP-2 complex and MMP-3

The amount of the 45 kDa MMP-3 was determined by titration with TIMP-1. The amount of TIMP-1 was calculated using $A_{280}^{1\%,1\ cm}$ of 10. The amount of the proMMP-2–TIMP-2 complex was determined by titration with MMP-3 on the basis that TIMP-2 in the complex is able to inhibit an active MMP in a 1:1 stoichiometry. The molar ratio of proMMP-2 to TIMP-2 of the complex was estimated to be 1:1 by measuring the amount of proMMP-2 after SDS/PAGE and staining with Coomassie Brilliant Blue R-250, comparison being made with known amounts of free proMMP-2 and TIMP-2 as standards.

Electrophoresis and zymography

SDS/PAGE was performed as described by Bury [33], and proteins were stained with Coomassie Brilliant Blue R-250. Zymography was conducted with SDS/polyacrylamide gels containing gelatin (0.8 mg/ml) as described by Hibbs et al. [34]. Samples were mixed with the SDS/PAGE sample buffer without a reducing agent and then subjected to electrophoretic analysis at room temperature. Enzymic activity was revealed by negative staining with Coomassie Brilliant Blue R-250.

Autoradiography

The iodo[^{14}C]acetamide-labelled samples were subjected to SDS/PAGE under non-reducing conditions. The gel was dried and exposed to a storage phosphor plate for 2 days [35]. The plate was then scanned using the Molecular Dynamics 400A PhosphorImager.

N-terminal sequence analysis

Automated sequence analysis was performed with an Applied Biosystems 477A pulse-liquid-phase sequencer with 'on-line' 120A phenylthiohydantoin (PTH)-amino acid analysis. To determine the N-terminal sequence of MMP-2 generated by MMP-3, the proMMP-2–TIMP-2 complex (1.8 nmol) was treated with 1 mM APMA at room temperature for 24 h and then allowed to react with 0.9 nmol of 45 kDa MMP-3 at 37 °C for 6 h. After stopping the reaction with 20 mM EDTA, the sample was applied to an SDS/PAGE [7.5% (w/v) total acrylamide] gel under reducing conditions and then transferred from the polyacrylamide gel to poly(vinylidene difluoride) membrane (PVDF) (PVDF-Millipore Immobilon transfer membrane) as described by Matsudaira [36]. Proteins transferred to the PVDF membrane were located by staining with Coomasie Brilliant Blue R-250, and the band of interest was excised, placed directly on to a Polybrene-treated glass filter, and sequenced. Net yields of amino acids were calculated by subtraction of the amount of PTH derivatives in each cycle from background in the previous cycle.

RESULTS

Preparation of the proMMP-2–TIMP-2 complex and proMMP-2 free of TIMP-2

Human uterine cervical fibroblasts secrete proMMP-2 and TIMP-2. When the conditioned medium was applied to a gelatin–Sepharose 4B column, a mixture of proMMP-2 and the proMMP-2–TIMP-2 complex was eluted from the column with

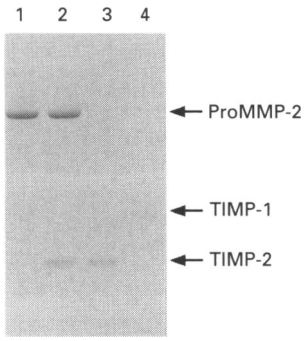


Figure 1 SDS/PAGE analysis of the proMMP-2–TIMP-2 complex

ProMMP-2 and the proMMP-2–TIMP-2 complex purified from the culture medium of human uterine cervical fibroblasts were analysed by SDS/PAGE under reducing conditions. Lane 1, proMMP-2; lane 2, proMMP-2–TIMP-2 complex; lane 3, TIMP-2; lane 4, TIMP-1. The gel was stained with Coomassie Brilliant Blue R-250.

5% dimethyl sulphoxide. This sample was then applied to a gel-permeation column of Sephacryl S-200 to separate the two components: the earlier fractions contained the proMMP-2–TIMP-2 complex (Figure 1, lane 2), while the later ones contained free proMMP-2 (Figure 1, lane 1). Pooled fractions of the complex contained neither free proMMP-2 nor free TIMP-2. This was concluded from results indicating a complete loss of MMP inhibitory activity from the complex and the lack of proMMP-2 processing when the preparation was activated with APMA (see below).

Activation of the proMMP-2–TIMP-2 complex by APMA

When proMMP-2 was treated with 1 mM APMA at 37 °C, it was activated fully within 1 h, and the activity decreased upon a longer incubation, as reported previously [6] (Figure 2). SDS/PAGE analysis indicated that the 72 kDa proMMP-2 was converted into a 65 kDa protein, but was further degraded (Figure 3a). In contrast, when the proMMP-2–TIMP-2 complex was treated with APMA, no gelatinolytic activity was detected (Figure 2). SDS/PAGE analysis of these samples showed that proMMP-2 in the complex was not converted into a lower-molecular-mass form even after a 24 h treatment (Figure 3a).

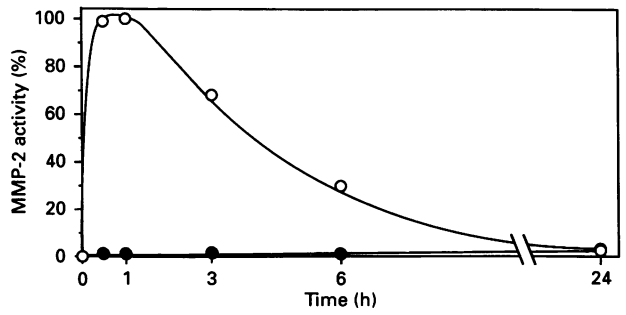


Figure 2 Lack of enzymic activity of the APMA-treated proMMP-2–TIMP-2 complex

ProMMP-2 (20 μg/ml) (○) or the proMMP-2–TIMP-2 complex (26 μg/ml) (●) was treated with 1 mM APMA at 37 °C for the indicated periods of time, and the enzymic activity was measured using [^{14}C]acetylated gelatin.

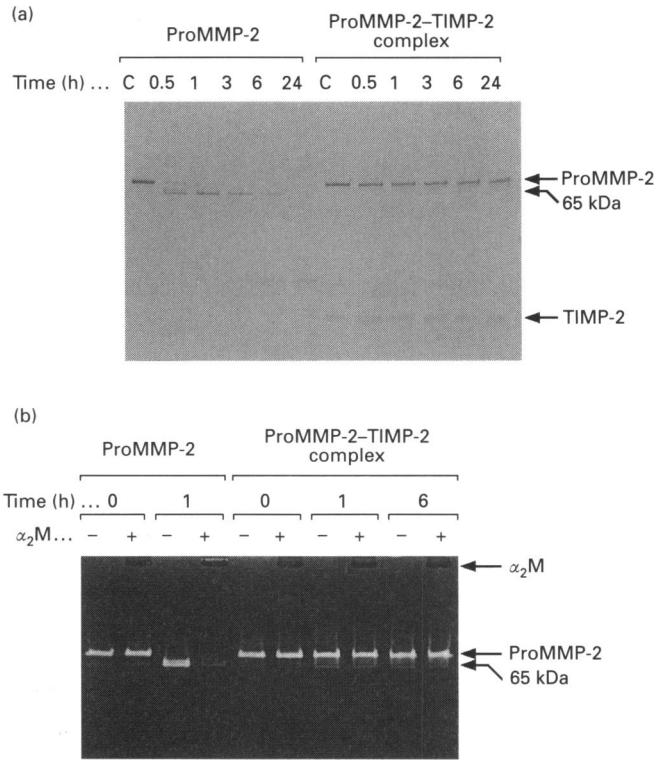


Figure 3 SDS/PAGE and zymographic analyses of the proMMP-2–TIMP-2 complex treated with APMA

(**a**) ProMMP-2 (20 μg/ml) or the proMMP-2–TIMP-2 complex (26 μg/ml) was incubated with 1 mM APMA at 37 °C for the indicated periods of time. The reaction was stopped by 20 mM EDTA, and the samples were analysed by SDS/PAGE under reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. Abbreviation: C, control. (**b**) ProMMP-2 (1 μg/ml) or the complex (1.3 μg/ml) was incubated with 1 mM APMA at 37 °C for the indicated periods of time. The samples were then divided into two halves and one portion was further allowed to react with α_2M (100 μg/ml) for 30 min at room temperature. Both halves of the samples were then analysed by zymography under non-reducing conditions.

These results contrast with those obtained previously, which showed gelatinolytic activity of the complex on APMA treatment [18–20,23–27]. To investigate further whether there was any proteolytically active MMP-2 generated during this treatment, the APMA-treated complex was incubated with human α_2M and the samples were subjected to zymographic analysis. α_2M is a 725 kDa tetrameric glycoprotein that binds and inhibits almost all endopeptidases [37], including MMPs [29,30,38]. The formation of a complex between α_2M and a proteinase is triggered by proteolytic attack of the enzyme on a specific region in the α_2M molecule [36,38,39]. When the APMA-treated proMMP-2 free of TIMP-2 was incubated with α_2M, the 68 and 65 kDa forms were entrapped by α_2M, and the bands corresponding to these positions shifted to the top of the gel as high-molecular-mass complexes (Figure 3b). However, when the APMA-treated proMMP-2–TIMP-2 complex was allowed to react with α_2M, there was no apparent shift of the gelatin-digestion zone, indicating that the complex was proteolytically inactive, even after APMA treatment. This suggests that proMMP-2 in the complex was transformed to an 'activated' proMMP-2 (proMMP-2*) by APMA, but it was immediately inhibited by TIMP-2 before the propeptide of proMMP-2* was processed. If this were the case, it could be anticipated that TIMP-2 in the proMMP-2*–TIMP-2 complex would no longer have MMP

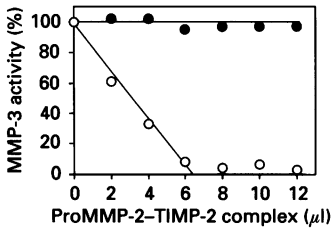


Figure 4 TIMP-2 activity in the complex before and after APMA treatment

The proMMP-2–TIMP-2 complex (20 μg/ml) was incubated with (●) or without (○) 1 mM APMA at 37 °C for 2 h and tested for its ability to inhibit MMP-3 (2.5 μg/ml). MMP-3 activity was measured using reduced [^{3}H]carboxymethylated transferrin as a substrate.

inhibition activity, owing to the newly arranged interaction of the N-terminal inhibitory domain of TIMP-2 and the exposed active site of proMMP-2*. To test this possibility, the inhibition activity of the complex against MMP-3 was measured before and after the APMA treatment. As shown in Figure 4, the proMMP-2–TIMP-2 complex inhibited MMP-3 in a 1:1 stoichiometry, but this activity was completely lost on treatment of the complex with APMA.

The activation mechanism of proMMPs by APMA has been proposed to occur through the reaction of APMA with the SH group of the cysteine residue in the propeptide when it is transiently dissociated from the Zn atom at the active site [9,10]. Recent studies by Chen et al. [40], however, have suggested that the initial 'trigger' in the activation of proMMP-3 by APMA is the induction of zymogen perturbation, which then promotes the dissociation of the Cys–Zn interaction and the subsequent reaction of APMA with the SH group of the cysteine residue. We therefore investigated whether or not the initial reaction of APMA with Cys^{73} in the propeptide of proMMP-2 is essential for zymogen binding to the N-terminal domain of TIMP-2. To examine this, the proMMP-2–TIMP-2 complex was treated with

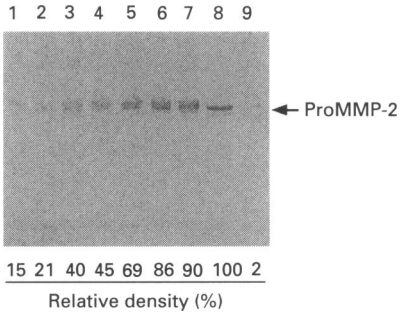


Figure 5 Labelling of Cys^{73} of proMMP-2 with iodo[^{14}C]acetamide during APMA treatment of the proMMP-2–TIMP-2 complex

The proMMP-2–TIMP-2 complex (100 μg/ml) was incubated with increasing concentrations of APMA in the presence of 1 mM iodo[^{14}C]acetamide at 37 °C for 1 h. After blocking free iodo[^{14}C]acetamide with 5 mM cysteine, the samples were subjected to SDS/PAGE under non-reducing conditions. The radioactivity incorporated into the 'APMA-activated' proMMP-2* was revealed and quantified by the phosphoImager. Lane 1, complex without APMA; lanes 2–7, complex treated with APMA (lane 2, 0.01 mM; lane 3, 0.05 mM; lane 4, 0.1 mM; lane 5, 0.2 mM; lane 6, 0.5 mM; lane 7, 1.0 mM); lane 8, complex treated with 20 mM EDTA; lane 9, sample in lane 5 that was further treated with 5 mM unlabelled iodoacetamide to block free cysteine, then allowed to react with 100 μg/ml 45 kDa MMP-3 (2:1 molar ratio) at 37 °C. The relative intensity of the radioactivity incorporated into proMMP-2* is shown, taking that of the EDTA-treated sample as 100%.

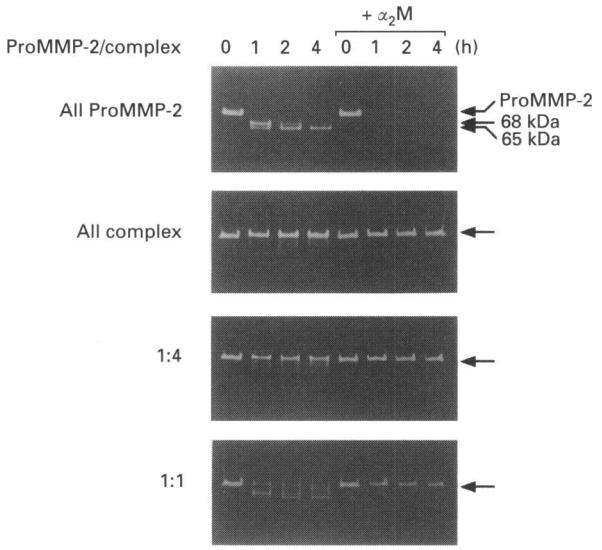


Figure 6 Zymographic analysis of the proMMP-2–TIMP-2 complex treated with APMA in the presence of free proMMP-2

The proMMP-2–TIMP-2 complex and free proMMP-2 were mixed at the molar ratios indicated, and the mixtures were treated with 1 mM APMA in the absence or presence of α_2M for the indicated periods of time at 37 °C. The samples were subjected to zymography under non-reducing conditions.

various concentrations of APMA in the presence of 1 mM iodo[^{14}C]acetamide at 37 °C for 1 h. After blocking free iodo-[^{14}C]acetamide with 5 mM cysteine, the samples were subjected to SDS/PAGE under non-reducing conditions, and radioactivity was detected using the phosphoImager [35]. As shown in Figure 5, proMMP-2* was labelled with iodo[^{14}C]acetamide in an APMA-concentration-dependent manner. At a lower concentration of APMA, labelling of proMMP-2* with iodo[^{14}C]-acetamide increased on a longer incubation (results not shown). On the other hand, when the proMMP-2–TIMP-2 complex was first allowed to react with 1 mM APMA for 1 h and then with 1 mM iodo[^{14}C]acetamide for 1 h at 37 °C, proMMP-2* was not radiolabelled (results not shown), indicating that the reaction of iodo[^{14}C]acetamide with proMMP-2* did not result from the replacement of the APMA bound to proMMP-2*. When the iodo[^{14}C]acetamide-labelled complex was treated with MMP-3 (Figure 5, lane 9), proMMP-2* was converted into the 65 kDa species (see also Figure 7 below). This latter form lacked radioactivity, suggesting that Cys^{73} in the propeptide was specifically labelled by iodo[^{14}C]acetamide. These results support the notion that the activation of proMMP-2 by APMA is triggered by perturbation of the zymogen, probably by binding through sites other than Cys^{73}.

Activation of the proMMP-2–TIMP-2 complex in the presence of free proMMP-2

In contrast with our observations, it has been reported that the 72 kDa proMMP-2 in the complex can be converted into an active 65 kDa form by APMA treatment, and the activated complex expresses a reduced gelatinolytic activity [18–20,23–27]. This might have been due to the presence of a small amount of free proMMP-2 in the preparation of the complex. To test this possibility, the complex was mixed with free proMMP-2 at various molar ratios and subjected to APMA activation. As shown in Figure 6, proMMP-2 in the complex, as well as free

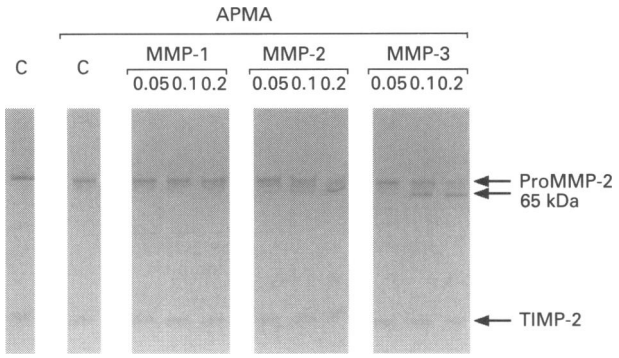


Figure 7 Molecular-mass conversion of proMMP-2 of the APMA-treated complex by MMP-2 or MMP-3

The proMMP-2–TIMP-2 complex (20 μg/ml) was first treated with 1 mM APMA for 1 h at 37 °C and then with the 41 kDa MMP-1, MMP-2 or the 45 kDa MMP-3 at a 0. 05:1, 0.1:1 or 0.2:1 molar ratio for 2 h at 37 °C. After terminating the reaction with 20 mM EDTA, the samples were subjected to SDS/PAGE under reducing conditions and the proteins were detected by immunoblotting using rabbit anti-(human MMP-2) serum and sheep anti-(human TIMP-2) serum. Abbreviation: C, control.

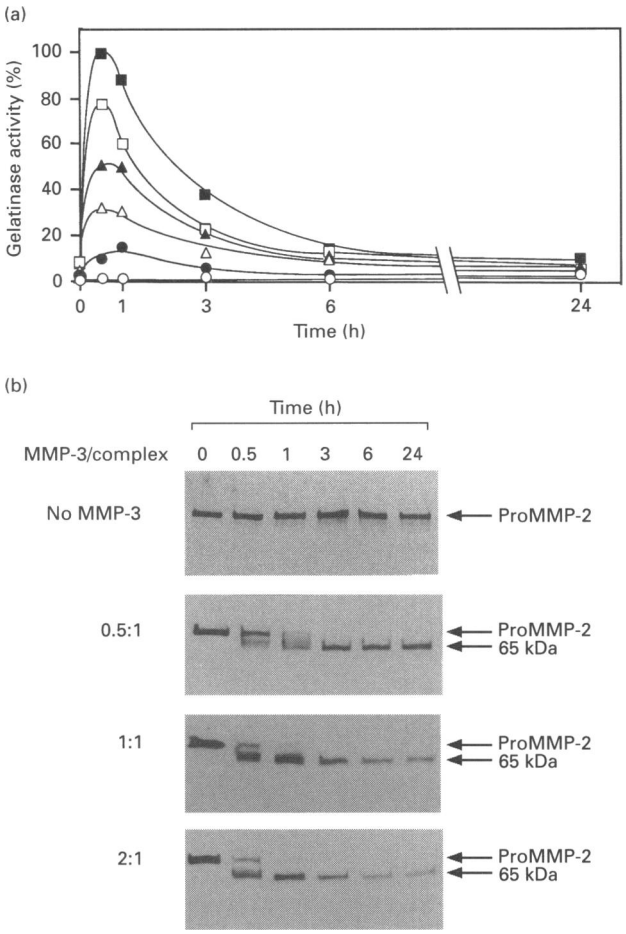


Figure 8 Activation of the proMMP-2–TIMP-2 complex by APMA in the presence of the 45 kDa MMP-3

The proMMP–2-TIMP-2 complex (20 μg/ml) was allowed to react with MMP-3 at molar ratios of 1:0.25 (●), 1:0.5 (△), 1:0.75 (▲), 1:1 (□), 1:2 (■) or without MMP-3 (○), and treated with 1 mM APMA at 37 °C for the indicated time periods. The samples were then subjected to (**a**) gelatinase assay, and (**b**) Western-blotting analysis using rabbit anti-(human MMP-2) serum for selected samples treated with MMP-3 at the indicated molar ratios.

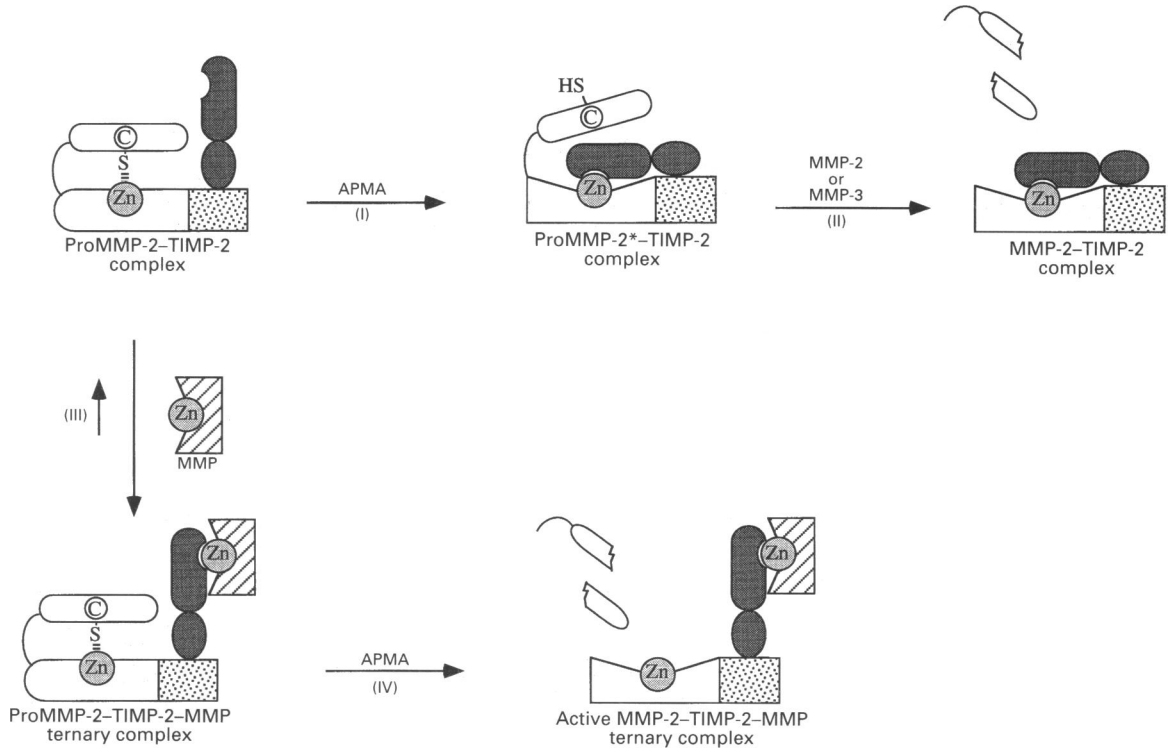
proMMP-2, was converted into three distinct forms, of molecular mass 70, 68 and 65 kDa, by APMA treatment, and the rate of conversion was dependent on the amount of free proMMP-2. When the mixtures were treated with APMA in the presence of α_2M, this conversion did not occur. This probably resulted from a rapid entrapment of the APMA-activated free MMP-2 by α_2M; therefore the conversion of proMMP-2 into the complex could not proceed. Indeed, the levels of the 72 kDa band detected after APMA activation in the presence of α_2M corresponded to the amount of the proMMP-2–TIMP-2 complex in the mixture (Figure 6). This indicates that the presence of free proMMP-2 promotes the conversion of proMMP-2 in the complex into lower-molecular-mass forms on APMA treatment. Two possible mechanisms could explain this phenomenon. First, the activated free MMP-2 rapidly binds to and removes TIMP-2 from the complex, and the released proMMP-2 is subsequently activated by APMA. Repetition of this 'cycle' would eventually allow all proMMP-2 in the complex to be converted into the 65 kDa form. However, this scheme seems unlikely, since the 'activated' proMMP-2* in the complex rapidly binds to TIMP-2 through the active site, and the TIMP-2 in this complex is no longer inhibitory (see Figure 4). A second possibility is that the activated free MMP-2 cleaves the propeptide from the 'activated' proMMP-2* that is bound to TIMP-2. To test this possibility, the APMA-activated complex was treated with a catalytic amount of MMP-2 and the processing of the proMMP-2* was analysed by SDS/PAGE. As shown in Figure 7, a catalytic amount of MMP-2 converted the proMMP-2* in the APMA-treated complex in a dose-dependent manner, indicating that the conversion of proMMP-2 of the complex into the 65 kDa species is due to the action of free MMP-2 on the proMMP-2*–TIMP-2 complex. This conversion was also observed with a catalytic amount of MMP-3, but not with MMP-1 (Figure 7). The N-terminal sequence analysis of the 65 kDa species generated by MMP-3 was Tyr-Asn-Phe-Phe-Pro, indicating that the Asn^{80}–Tyr^{81} bond had been cleaved. Although the proMMP-2* in the complex was converted into the 65 kDa species by MMP-2 or MMP-3, it did not exhibit gelatinolytic activity (results not shown).

Activation of the proMMP-2–TIMP-2 complex by APMA in the presence of MMP-3

While the APMA-treated proMMP-2–TIMP-2 complex did not exhibit enzymic activity, proMMP-2 in the complex became proteolytically active when it was allowed to react with active MMP-3 and then treated with APMA (Figures 8a and 8b). The level of gelatinolytic activity detected was dependent on the MMP-3/complex molar ratio. This suggests that coupling of TIMP-2 in the complex with another active MMP molecule (e.g., MMP-3) is essential for the expression of MMP-2 activity.

DISCUSSION

MMPs are synthesized and secreted from cells to the extracellular space as proenzymes. While the exact activation mechanisms of these zymogens *in vivo* are not well understood, proMMPs are activated *in vitro* by treatment with proteinases, mercurial compounds, chaotropic agents, HOCl, low pH and heat [9,10,25,41,42]. However, the finding that TIMP-2 can form a complex with proMMP-2 has added more complexity to the



Scheme 1 Steps involved in activation of the proMMP-2–TIMP-2 complex by APMA

(I) The treatment of the proMMP-2–TIMP-2 complex with APMA induces conformational changes in proMMP-2 and dissociates the Zn–Cys interaction, thereby allowing the active site of the zymogen to react with the N-terminal inhibitory domain of TIMP-2. The propeptide therefore is not processed and the activated proMMP-2*–TIMP-2 complex is proteolytically inactive. (II) Treatment of the proMMP-2*–TIMP-2 complex with MMP-2 or MMP-3 removes the propeptide, but the complex remains inactive. (III) An active MMP binds and blocks the N-terminal domain of TIMP-2 in the complex. (IV) ProMMP-2 of the ternary complex is then activated by APMA. A majority (> 90%) of the APMA-activated MMP-2 remains as a ternary complex of MMP-2–TIMP-2–MMP. The level of MMP-2 activity attained depends on the amount of active MMP.

known activation mechanisms of proMMP-2, because TIMP-2 in the complex not only inhibits the active MMPs, but also affects its activation processes. Here we have investigated a possible mode of activation of the proMMP-2–TIMP-2 complex by APMA and the fate of TIMP-2 in the complex after activation.

It has been reported that TIMP-2 which is bound to proMMP-2 does not inhibit the conversion and the activation of proMMP-2 by APMA, and the APMA-activated complex exhibited a lower gelatinolytic activity compared with that of free MMP-2 [18–20,23–27]. The requirement of a further addition of a stoichiometric amount of TIMP-2 to inhibit the MMP-2 activity of the complex supported this notion [19,26]. On the other hand, Fridman et al. [27] speculated that the low activity in the APMA-treated complex might be due to contamination by free proMMP-2. However, this possibility was not investigated. In the present study we were able to isolate the proMMP-2–TIMP-2 complex virtually free of unbound proMMP-2 and TIMP-2. Using this preparation, we have demonstrated that the treatment of the complex with APMA rearranges the interaction between TIMP-2 and proMMP-2 and that the N-terminal domain of TIMP-2 reacts with the catalytic site of the 'activated' proMMP-2* before proMMP-2* loses its propeptide by autolysis (Scheme 1, step I). This mode of the action is further supported by the observations that the inhibitory activity of TIMP-2 in the 'virgin complex' is lost after APMA treatment and the SH group of Cys^{73} in the propeptide becomes available for alkylation. The proMMP-2*–TIMP-2 complex exhibits no gelatinolytic activity. It is, however, not known whether the formation of proMMP-2*–TIMP-2 complex is formed as an intra- or inter-complex rearrangement. In the case of inter-complex rearrangement, the polymerization of $(proMMP\text{-}2^*\text{–}TIMP\text{-}2)_n$ is anticipated, but gel-permeation chromatography of the proMMP-2–TIMP-2 complex and the proMMP-2*–TIMP-2 complex showed that both samples were eluted at essentially the same position, with $K_{av.}$ values of 0.44 and 0.46 respectively (results not shown). This suggests that the rearrangement probably occurs within a single complex.

We have previously shown that disruption of the Cys^{75} and Zn co-ordination is essential for activation of proMMP-3, but insufficient to cause activation [40]. Therefore it was concluded that the activation of proMMP-3 by APMA is initiated by molecular perturbation of the zymogen [40]. A similar mechanism seems to be employed in the activation of proMMP-2 by APMA. The fact that Cys^{73} was alkylated with iodo[^{14}C]acetamide during APMA treatment suggests that the 'trigger' for the activation of proMMP-2 by APMA is not the reaction of APMA with Cys^{73}, but is the initiation of perturbation in the proMMP-2 molecule. The conformational changes in proMMP-2 subsequently disrupt the Cys^{73}–Zn interaction. This then allows TIMP-2 in the complex to interact with the exposed active site of proMMP-2*. Our studies indicate that the previously reported low gelatinolytic activity detected with the APMA-treated complex is not derived from the activated complex, but it is most likely attributed to a small amount of free proMMP-2 present in the preparation.

It was also reported that the treatment of the proMMP-2–TIMP-2 complex with APMA results in conversion of 72 kDa

proMMP-2 into the 65 kDa species which corresponds to the active form of MMP-2 [19,24–26]. We have demonstrated that this conversion is generated by the proteolytic action of free MMP-2 on the proMMP-2*–TIMP-2 complex (Scheme 1, step II). The conversion of proMMP-2* into the 65 kDa form did not reveal proteolytic activity, since the enzyme remained tightly bound to the inhibitor. Interestingly, similar processing of proMMP-2* in the complex occurred with a catalytic amount of MMP-3 by cleaving the Asn^{80}–Tyr^{81} bond, the site cleaved by APMA treatment of proMMP-2 [6,11]. However, neither MMP-2 nor MMP-3 could attack this bond of the native proMMP-2, suggesting that conformational changes of the propeptide occur on APMA treatment.

Only free proMMP-2 expresses enzymic activity on APMA activation. However, gelatinolytic activity is detected when the complex is first allowed to react with an active MMP-3 and then with APMA (Scheme 1, steps III and IV). The levels of activation attained by this procedure correlated with the amount of MMP-3 (Figure 8). After APMA treatment, as reported by Kolkenbrock et al. [43], more than 90% of activated MMP-2 remained as a ternay complex (results not shown). Miyazaki et al. [44] reported that MMP-3 is an activator of the proMMP-2 that is bound to TIMP-2. As described above, MMP-3 does not directly activate proMMP-2. MMP-3 assists the APMA activation of proMMP-2 in the complex by blocking the inhibitory domain of TIMP-2. Similar results are likely to be obtained with other active MMPs. The availability of active MMPs also affects the activation of the proMMP-2–TIMP-2 complex on the cell surface. The C-terminal domain of proMMP-2 is required for the activation of proMMP-2 by the plasma-membrane activator [16,21,45], which is most likely to be the recently discovered MT-MMP [17]. An excess amount of C-terminal domain of MMP-2 or the association of TIMP-2 to proMMP-2 through their C-termini inhibits this process [16,45]. However, the interaction of TIMP-2 in the complex with an active MMP allowed proMMP-2 to be activated by the membrane (Y. Itoh and H. Nagase, unpublished work). This emphasizes that not only the balance between proMMP-2 and TIMP-2 production, but also the level of other active MMPs, would dictate the MMP-2 activity. Such a mechanism may participate in tuning precise catabolic processes of the extracellular matrix components *in vivo*.

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